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ORIGINAL ARTICLE

Evaluation of the efficacy of electrochemically activated solutions against nosocomial pathogens and bacterial endospores

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Abstract

Aims: Electrochemically activated solutions (ECAS) are generated from halide salt solutions via specially designed electrolytic cells. The active solutions are known to possess high biocidal activity against a wide range of target microbial species, however, literature revealing the kill-kinetics of these solutions is limited. The aim of the study was to identify the kill-rate and extent of population kill for a range of target species (including endospores) using ECAS generated at the anode (anolyte).

Methods and Results: Standard suspensions of methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus atrophaeus* spores and *Clostridium difficile* spores were treated with anolyte in a quantitative suspension assay. For vegetative cells, all concentrations of anolyte tested reduced the viable population to below the detection limit within 10 s. At a concentration of 99%, anolyte produced a $\log_{10}$ reduction factor of greater than five in viable *B. atrophaeus* endospores within 90 s and reduced numbers of *C. difficile* endospores to below the experimental detection limit within 20 s at concentrations of 5% or greater.

Conclusions: Anolyte was highly effective in killing test-bacteria and spores. The bactericidal efficacy was retained against vegetative cells at dilutions as low as 1% and against *C. difficile spores* as low as 5%.

Significance and Impact of Study: The results of this study demonstrate that ECAS are effective at lower concentrations and act more rapidly than previously reported. Potent bactericidal and sporicidal activity coupled with point-of-use generation, low production-costs and environmental compatibility suggest that acidic ECAS has the potential to be a useful addition to the current armoury of disinfectants.

Introduction

The appropriate and effective use of disinfectants is necessary to limit the spread of infectious or transmissible agents. Electrochemically activated solutions (ECAS) may represent a viable alternative to commonly used disinfectant solutions and a multitude of systems for the generation of active killing solutions *in situ* using electrochemical cells have been described. The physicochemical nature of ECAS is poorly understood. The solutions exhibit high redox potentials, contain free radical species and are thought to exist in a metastable state. Anolyte (the product of the anode chamber) has a high oxidation potential of around +1000 mV, whereas catholyte (the product of the cathode chamber) has a high reduction potential of around −800 mV. Following production, the solutions remain metastable for *c.* 48 h before returning to a stable, inactive state. It is the solution produced at the anode which possesses high-level antimicrobial properties (Marais and Brozel 1999).

Systems developed in Russia produce ECAS from 'tap water' and saline solution via a flow-through electrolytic module (FEM), comprising coated titanium electrodes that are separated by an electro-catalytic ceramic diaphragm. ECAS produced from these systems have been used extensively for washing fruit and vegetables, cooling tower disinfection, swimming pool sanitation, dermatological washing, cleaning and dressing of wounds and disinfection of instruments (Leonov 1997).

Other ECAS systems, such as Sterilox® (PuriCore, Malvern, PA), exhibit oxidation potentials of >+950 mV and a pH between 5·0 and 6·5. Known active chemical species include hypochlorous acid (144 mg l^{-1}), free chlorine and a variety of (nondefined) oxidizing substances (Selkon *et al.* 1999). Sterilox® has been reported to exhibit rapid and potent biocidal activity against a range of microorganisms including endospores (Shetty *et al.* 1999).

A system producing 'electrolysed oxidizing (EO) water' (Sharma and Demirci 2003) by the electrolysis of dilute saline solution and has been widely adopted in Japan where it is used as a disinfectant in hospitals and dental clinics (Al-Haq *et al.* 2002). EO water has demonstrated biocidal activity against a range of pathogens (Kim *et al.* 2000) and has been shown to dramatically reduce the onset of spoilage when used as a surface sanitizer for fruit, vegetables and fish (Al-Haq *et al.* 2002; Ozer and Demirci 2006). An alternative ECAS, 'mixed-oxidant solutions' (MIOX) achieved a >99·9% inactivation of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores which are normally recalcitrant to chlorine and other drinking-water disinfectants (Venczel *et al.* 1997).

The ECAS evaluated in this study are produced by electrolysis of sodium chloride solution in a flow-through electrolytic cell system containing a novel Ti/SnO_2-IrO_2-TiO_2 anode. The ECAS generated at this anode have been shown to contain a variety of oxidants including hydroxyl radical, hypochlorous acid, chlorine and related intermediate species (Cai 2005) with a redox potential of *c.* +1180 mV at a pH of 2·2 making the test-anolyte among the most oxidizing so far described.

This study investigates the efficacy of anolyte when used to challenge four bacterial species in a quantitative suspension test. The purpose was to establish the efficacy of anolyte for use as a disinfectant which can be produced on demand at the point of use. Two vegetative species, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*, along with spores of aerobic (*Bacillus atrophaeus*) and anaerobic (*Clostridium difficile*) spore-forming bacteria were challenged with anolyte. Previous studies have investigated the efficacy of ECAS against a range of organisms but have been limited to a single concentration of ECAS (Tanaka *et al.* 1996; Landa-Solis *et al.* 2005) or have not sampled within the first minute of contact with disinfectant (Shetty *et al.* 1999; Loshon *et al.* 2001). It has been reported, however, that these solutions are highly effective over time intervals as short as 10 s (Tanaka *et al.* 1996) therefore this study was designed to investigate the efficacy of a range of concentrations of ECAS over a 90 s time period with short sampling intervals, particularly during the early phase of treatment.

Materials and methods

Bacterial strains and growth conditions

MRSA (SMH 11622 MRSA, kind gift from Southmead Hospital, Bristol, UK), *P. aeruginosa* (PAO1 SE1, University of the West of England, Bristol, UK) and *B. atrophaeus* (NCIMB 8649) were cultured on nutrient agar (Oxoid Ltd, Basingstoke, UK). For broth culture, single, isolated colonies were subcultured into nutrient broth (Oxoid Ltd) and grown at 37°C shaking at 200 rev min^{-1}. *C. difficile* (NCIMB 10666) was incubated at 37°C anaerobically (MK3 Anaerobic work station, Don Whitley Scientific, Shipley, UK) on Reinforced Clostridial Agar (Oxoid Ltd) supplemented with 5% sterile defibrinated horse blood (TCS Biosciences, Buckingham, UK). *B. atrophaeus* and *C. difficile* were grown (20 plates per species) for 48 and 72 h, respectively. *B. atrophaeus* was scraped from the plates using sterile plastic blades and emulsified in 20 ml sterile ¼ strength Ringer's Solution (Oxoid Ltd). The suspension was centrifuged at 3000 ***g*** for 15 min [ALC PK121R, Jouan, Cologno Monzese (MI), Italy] and the pellets resuspended in sterile ¼ strength Ringer's solution, then washed and heat treated at 70°C for 10 min to kill remaining vegetative organisms, confirmed by resuspending aliquots in 95% ethanol and conducting comparative viable counts. Following anaerobic incubation, *C. difficile* was incubated aerobically for 24 h at room temperature to encourage sporulation before being emulsified in 20 ml of 50% ethanol to kill remaining vegetative cells.

Production of ECAS

Anolyte was generated by the electrolysis of 10% (w/v) NaCl solution using an electrochemical system supplied by Purest Solutions Ltd (London, UK). The electrochemical cell was flushed with deionized water. ECAS was produced by activating the saline pump and applying a potential across the cell. Cell current was maintained at 7·5 amps during collection. The redox potential and pH of the anolyte were measured immediately after collection (Sartorius PT-10P portable meter with PYP12 pH electrode and CEPTRL/87 ORP electrode; Sartorius, Epsom,

UK). The pH of all solutions was <2·46 and the redox potential >+1170mV.

Bactericidal/sporicidal assessment of anolyte

Quantitative suspension tests adapted from Suller and Russell (2000) were performed to assess the bactericidal efficacy of ECAS. For MRSA and *P. aeruginosa*, overnight cultures were subcultured 1 : 100 in to fresh, prewarmed nutrient broth in a 300-ml Erlenmeyer flask and grown to an optical density (OD_{600nm}) of 0·5. One hundred millilitres of culture was centrifuged at 3000 ***g*** for 15 min, washed twice in an equal volume of sterile ¼ strength Ringer's Solution before resuspension in 10 ml sterile ¼ strength Ringer's solution. One millilitre of the bacterial suspension was added to sterile distilled water in a 300-ml Erlenmeyer flask, and the appropriate volume of anolyte added to achieve the desired concentration in a total volume of 100 ml with an initial inoculum of *c.* 10^7 CFU ml^{-1}. *B. atrophaeus* and *C. difficile* endospore inocula were *c.* 1×10^8 CFU ml^{-1} and 1×10^5 CFU ml^{-1}, respectively. After time intervals of 10, 20, 30, 45, 60 and 90 s, 0·5-ml samples were removed to 4·5 ml of neutralizing diluent (foetal bovine serum). Efficacy of neutralizer was confirmed by adding 0·5 ml of 100%, 10%, 1% ECAS or a ¼ strength Ringer's solution control to 4·5-ml serum, mixing and immediately removing aliquots to tubes containing MRSA at 10^7 CFU ml^{-1}. The concentration of ECAS added had no significant impact on the number of survivors compared to the control ($P = 0{\cdot}9745$, 99% CI, data not shown). Triplicate count recovery plates were produced (50 μl deposited volume) using a Whitley Automatic Spiral Plater (Don Whitley Scientific, Shipley, UK) and incubated under appropriate conditions for 24–48 h. Colonies were counted and the number of survivors expressed as CFU ml^{-1}. The experimental detection limit was defined by the tenfold dilution into neutralizer and deposited volume of the spiral plater as 2×10^2 CFU ml^{-1}. Kill curves were constructed by plotting $\log_{10}$ number of survivors against time to determine the extent of kill in terms of $\log_{10}$ reduction factor and corresponding decimal reduction times (D-value). The kill-rate (K) is expressed as the $\log_{10}$ reduction factor min^{-1}.

Results

For vegetative cells, very rapid killing was observed. At all concentrations tested, anolyte reduced bacterial numbers to below the detection limit within 10 s (Fig. 1a,b) corresponding to a $\log_{10}$ reduction factor of ≥4·807 and ≥5·049 (within 10 s) for MRSA and *P. aeruginosa,* respectively. The D-values were not more than 2·08 s for MRSA and 1·98 s for *P. aeruginosa* after 10 s exposure to anolyte at concentrations of 1% or greater.

Bacterial endospores were substantially more resistant to the activity of anolyte than vegetative cells. When used to challenge spores of *B. atrophaeus* (Fig. 1c) at concentrations of 10% or lower, the sporicidal effect was not statistically significant ($P = 0{\cdot}42$, 99% CI) over 90 s. At 25% v/v, anolyte achieved a $\log_{10}$ reduction factor in viable spores of 3·605 ($K = 2{\cdot}4$) and for a concentration of 99%, 5·568 ($K = 3{\cdot}7$). This corresponds to D-values of 25 and 16·2 s for 25% and 99% anolyte, respectively. Against *C. difficile* (Fig. 1d), anolyte at 1% did not produce a statistically significant reduction in viable spores ($P = 0{\cdot}27$, 99% CI). At 5%, however, anolyte resulted in a $\log_{10}$ reduction factor of 2·562 ($K = 15{\cdot}4$) within the first 10 s dropping below the experimental detection limit by 20 s with a corresponding D-value of 3·9 s. At 10% or greater, the number of viable spores dropped below the experimental detection limit within 10 s corresponding to a $\log_{10}$ reduction factor of ≥2·714 and a D-value of not more than 3·68 s.

Discussion

When used appropriately, disinfectants are generally effective; however, their use raises a number of concerns regarding their toxicity, cost, stability over time and environmental impact. Electrochemically activated solutions have the potential to address many of these issues and there is a growing body of evidence that they are highly effective, low-cost, environmentally compatible microbicidal agents.

This study has demonstrated that anolyte is a highly effective disinfectant with potent bactericidal and sporicidal activity which is retained at very low concentrations for vegetative cells. Furthermore, we have demonstrated that the rapid bactericidal activity of these solutions is evident even when diluted as much as 100-fold.

A 'superoxidized water' with an oxidation potential of +1000–1100 mV and a pH of 2·3–2·7 was used against vegetative species including MRSA and *P. aeruginosa* (Tanaka *et al.* 1996). A reduction in viable counts was observed to below the level of detection within 10 s for all species tested (with the exception of *Burkholderia cepecia*) using an inoculum of $1{\cdot}7 \times 10^6$ CFU ml^{-1} and a 5 : 1 ratio of undiluted superoxidized water to inoculum. This is consistent with our findings, however, our initial inoculum was higher and we demonstrated that the efficacy of ECAS against vegetative cells is retained at concentrations as low as 1%. Tanaka *et al.* concluded that the activity of 'superoxidized water' was equivalent to 80% ethanol and superior to that of 0·1% chlorhexidine or 0·02% povidone iodine.

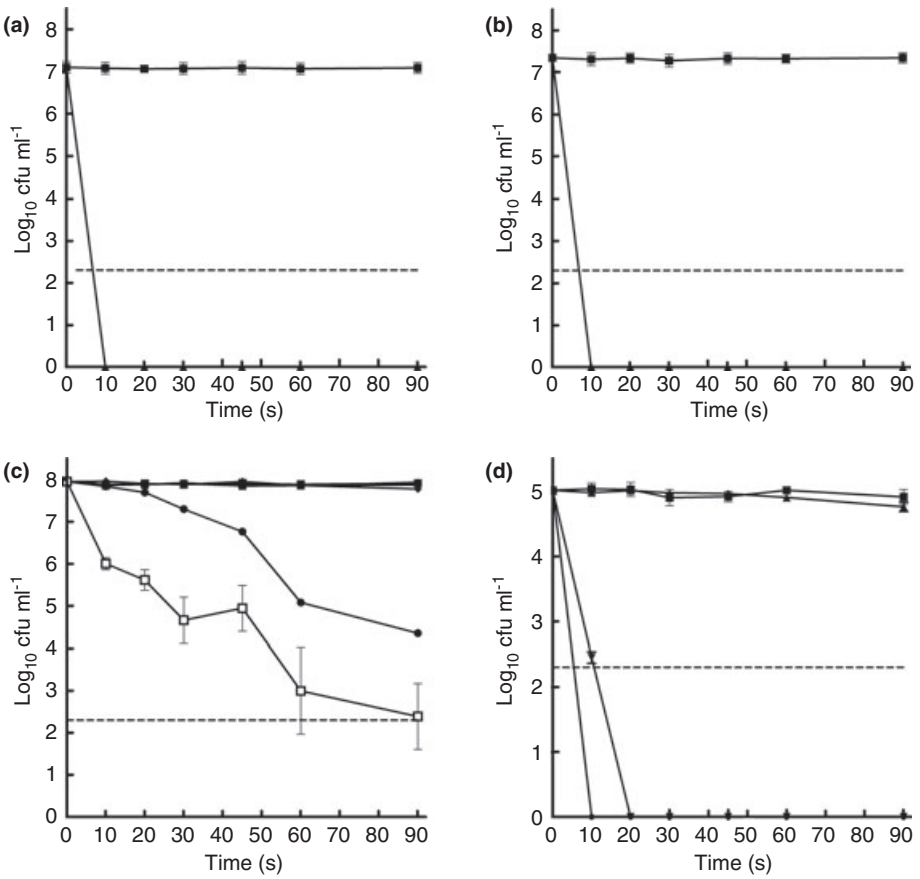

Figure 1 Antibacterial activity of 1% (▲), 5% (▼), 10% (♦), 25% (●) and 99% (□) anolyte compared to the control (■) against a) methicillin-resistant *Staphylococcus aureus*, b) *Pseudomonas aeruginosa*, c) *Bacillus atrophaeus* endospores, d) *Clostridium difficile* endospores over a period of 90 s. The detection limit of the assay is shown (- - - -). Concentrations ≥1% (for MRSA and *P. aeruginosa*) and ≥10% for *C. difficile* reduced the number of viable survivors to below the detection limit within 10 s therefore the higher concentrations are omitted for clarity.

Landa-Solis *et al.* (2005) tested the ECAS Microcyn™ (Oculus Innovative Sciences, Petaluma, CA) against a range of vegetative bacterial species including *S. aureus* and *P. aeruginosa* reporting a $\log_{10}$ reduction factor of eight within 30 s when challenged with 90% Microcyn™. Our research has revealed that anolyte reduced viable vegetative bacteria to below the detection limit of our assay within 10 s and that the efficacy was retained at concentrations as low as 1%. Landa-Solis *et al.* also reported a $\log_{10}$ reduction factor of greater than four within 2 min for *B. atrophaeus* spores using 90% Microcyn™ when challenged with an inoculum of 10^7 CFU ml^{-1}. This is comparable to our observations which showed a 3·6 and 5·5 $\log_{10}$ reduction (within 90 s) using 25 and 99% anolyte and an inoculum of $\sim 10^8$ CFU ml^{-1}.

Spores of *B. atrophaeus* were selected for use in this assay as they are known to be highly resistant to inactivation by disinfectants (Sagripanti *et al.* 2007). A study investigating the effect of the ECAS Sterilox® on spores of *Bacillus subtilis* suggested that the mechanism of action was through oxidation of proteins or fatty acids of the inner-membrane of dormant spores resulting in membrane permeabilization upon germination (Loshon *et al.* 2001). Using undiluted Sterilox®, the authors observed a $\log_{10}$ reduction of greater than five over 5 min and a $\log_{10}$ reduction of two with 10% Sterilox®. Our research demonstrated that ECAS are capable of achieving reductions in viable spores more rapidly than previously reported in the literature. Liao *et al.* (2007) investigated the contribution of oxidation potential to the killing effect of 'electrolyzed oxidizing water' in *E. coli* concluding that it was responsible for damaging inner and outer bacterial membranes. Cloete *et al.* (2009) demonstrated that a similar anolyte killed *E. coli* and *P. aeruginosa* through degradation of proteins because of oxidative stress. The high oxidation potential of the test-anolyte (+1180 mV) could account for the high sporicidal activity and efficacy against vegetative cells observed at the very low concentrations used in our study.

Shetty *et al.* (1999) demonstrated a $\log_{10}$ reduction of >4 at the first sample point of 2 min when *C. difficile* was challenged with Sterilox® at a ratio of spore suspension to Sterilox® of 1 : 10. By sampling more frequently, we have demonstrated that the anloyte used in this study achieved a $\log_{10}$ reduction of >2·5 within the first 10 s of treatment when diluted to 5%.

Our results show that anolyte is a potent bactericidal and sporicidal disinfectant with unique properties, which make it an attractive alternative to many disinfectants currently in use. It has been noted that acidic super-oxidized waters (such as the test-anolyte) are active, though unstable microbicidal agents (Landa-Solis *et al.* 2005). However, stability becomes less important if active solutions can be generated when required. Production of the test-anolyte is designed to be at the point of use, removing the need for storage. There is growing concern about accumulation of biocides, which have long-lasting residual activity within the environment. Anolyte is readily deactivated when exposed to air for 24 h and the process can be accelerated by stirring the solution (Cai 2005).

Our findings have revealed more detail regarding the extent and rate of kill during the early phase of treatment using ECAS than has been previously published. Earlier work, which may have been limited in the frequency of sampling or the range of concentrations tested, supports our findings and a consistent trend throughout the published research involving ECAS, which is the speed at which these solutions have been shown to kill bacterial cells. The test-anolyte has demonstrated potent sporicidal activity against *C. difficile* endospores, reducing the number of viable spores to below the detection limit of the assay within 20 s at a concentration of 5%. The test-anolyte was also highly active against spores of *B. atrophaeus* and achieved reductions in viable spores more rapidly than previously reported. Against *P. aeruginosa* and MRSA, anolyte reduced the number of viable organisms to below the detection limit within 10 s at concentrations as low as 1%. Even at such low concentrations, determination of accurate kill-rates is not possible. Loshon *et al.* (2001) experienced similar difficulties when attempting to measure accurate killing rates using undiluted Sterilox®. Accurate determination of kill-rates using these solutions is hindered by the physical limitations of traditional microbiology in terms of sampling time. Novel techniques may be required to fully elucidate the rate and mechanism of kill of vegetative bacterial cells. Anolyte and acidic ECAS in general, could offer a safe, low-cost, point-of-use solution to address the problems associated with other fast-acting disinfectants in a range of applications.

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References

Al-Haq, M.I., Seo, Y., Oshita, S. and Kawagoe, Y. (2002) Disinfection effects of electrolyzed oxidizing water on suppressing fruit rot of pear caused by *Botryosphaeria berengeriana*. *Food Res Int* **35**, 657–664.

Cai, Z. (2005) *Characterisation of electrochemically activated solutions for use in environmental remediation*. PhD thesis. University of the West of England, Bristol, UK.

Cloete, T.E., Thantsha, M.S., Maluleke, M.R. and Kirkpatrick, R. (2009) The antimicrobial mechanism of electrochemically activated water against *Pseudomonas aeruginosa* and *Escherichia coli* as determined by SDS-PAGE analysis. *J Appl Microbiol* **107**, 379–384.

Kim, C., Hung, Y.-C. and Brackett, R.E. (2000) Efficacy of electrolyzed oxidizing (EO) and chemically modified water on different types of foodborne pathogens. *Int J Food Microbiol* **61**, 199–207.

Landa-Solis, C., Gonzalez-Espinosa, D., Guzman-Soriano, B., Snyder, M., Reyes-Teran, G., Torres, K. and Gutierrez, A.A. (2005) Microcyn: a novel super-oxidized water with neutral pH and disinfectant activity. *J Hosp Infect* **61**, 291–299.

Leonov, B.I. (1997) Electrochemical activation of water and aqueous solutions: past, present and future. In *Proceedings of the First International Symposium on Electrochemical Activation, Moscow*. pp. 11–27.

Liao, L.B., Chen, W.M. and Xiao, X.M. (2007) The generation and inactivation mechanism of oxidation-reduction potential of electrolyzed oxidizing water. *J Food Eng* **78**, 1326–1332.

Loshon, C.A., Melly, E., Setlow, B. and Setlow, P. (2001) Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox. *J Appl Microbiol* **91**, 1051–1058.

Marais, J.T. and Brozel, V.S. (1999) Electro-chemically activated water in dental unit water lines. *Br Dent J* **187**, 154–158.

Ozer, N.P. and Demirci, A. (2006) Electrolyzed oxidizing water treatment for decontamination of raw salmon inoculated with *Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A and response surface modeling. *J Food Eng* **72**, 234–241.

Sagripanti, J.L., Carrera, M., Insalaco, J., Ziemski, M., Rogers, J. and Zandomeni, R. (2007) Virulent spores of *Bacillus anthracis* and other *Bacillus* species deposited on solid surfaces have similar sensitivity to chemical decontaminants. *J Appl Microbiol* **102**, 11–21.

Selkon, J.B., Babb, J.R. and Morris, R. (1999) Evaluation of the antimicrobial activity of a new super-oxidized water, Sterilox, for the disinfection of endoscopes. *J Hosp Infect* **41**, 59–70.

Sharma, R.R. and Demirci, A. (2003) Treatment of *Escherichia coli* O157:H7 inoculated alfalfa seeds and sprouts with electrolyzed oxidizing water. *Int J Food Microbiol* **86**, 231–237.

Shetty, N., Srinivasan, S., Holton, J. and Ridgway, G.L. (1999) Evaluation of microbicidal activity of a new disinfectant: Sterilox 2500 against *Clostridium difficile* spores, *Helicobacter pylori*, vancomycin resistant *Enterococcus* species, *Candida albicans* and several *Mycobacterium* species. *J Hosp Infect* **41**, 101–105.

Suller, M.T. and Russell, A.D. (2000) Triclosan and antibiotic resistance in *Staphylococcus aureus*. *J Antimicrob Chemother* **46**, 11–18.

Tanaka, H., Hirakata, Y., Kaku, M., Yoshida, R., Takemura, H., Mizukane, R., Ishida, K., Tomono, K. *et al.* (1996) Antimicrobial activity of superoxidized water. *J Hosp Infect* **34**, 43–49.

Venczel, L.V., Arrowood, M., Hurd, M. and Sobsey, M.D. (1997) Inactivation of *Cryptosporidium parvum* oocysts and clostridium perfringens spores by a mixed-oxidant disinfectant and by free chlorine. *Appl Environ Microbiol* **63**, 4625.

